

## Critical Roles of TRAF2 and TRAF5 in Tumor Necrosis Factor-induced NF- $\kappa$ B Activation and Protection from Cell Death\*

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**Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) were identified as signal transducers for the TNF receptor superfamily. However, the exact roles of TRAF2 and TRAF5 in TNF-induced NF- $\kappa$ B activation still remain controversial. To address this issue, we generated TRAF2 and TRAF5 double knockout (DKO) mice. TNF- but not interleukin-1-induced nuclear translocation of NF- $\kappa$ B was severely impaired in murine embryonic fibroblasts (MEFs) derived from DKO mice. Moreover, DKO MEFs were more susceptible to TNF-induced cytotoxicity than TRAF2 knockout MEFs. Collectively, these results indicate that both TRAF2 and TRAF5 are involved in TNF-induced NF- $\kappa$ B activation and protection from cell death.**

Tumor necrosis factor (TNF)<sup>1</sup> exerts a variety of biological effects, including production of inflammatory cytokines, up-regulation of adhesion molecules, proliferation, differentiation, and apoptosis (1). Although such pleiotropic effects are mediated by two cognate TNF receptors, TNF-R1 and TNF-R2, TNF-induced cell death is mediated mainly by TNF-R1. In response to TNF, TNF-R1 is trimerized and recruits an adapter molecule, TRADD (2). In the apoptotic signaling pathway, the recruited TRADD interacts with FADD (Fas-associated death domain protein) (2), which then recruits and activates caspase-8. The activated caspase-8 in turn activates effector

caspases, such as caspase-3 and -7, resulting in apoptosis (3). On the other hand, recruited TRADD also interacts with RIP and TNF receptor-associated factor 2 (TRAF2), both of which are implicated in NF- $\kappa$ B and c-Jun N-terminal kinase (JNK) activation (4, 5).

NF- $\kappa$ B is a transcriptional factor that regulates expression of various inflammatory cytokines, chemokines, and adhesion molecules (6). NF- $\kappa$ B is activated by inflammatory cytokines and cellular stresses including TNF, IL-1, lipopolysaccharide, UV, or  $\gamma$ -irradiation. In unstimulated cells, NF- $\kappa$ B is sequestered in the cytoplasm by binding to I $\kappa$ B inhibitory proteins (7). Upon stimulation, N-terminal serine residues of I $\kappa$ Bs are phosphorylated, which leads to ubiquitination and subsequent degradation via a 26-S proteasome pathway (8). Then the liberated NF- $\kappa$ B from I $\kappa$ Bs translocates to the nucleus and activates transcription of various target genes. Recently, the I $\kappa$ B kinase (IKK) complex, which is responsible for this inducible phosphorylation, was identified and extensively characterized (9). The IKK complex is composed of three subunits, including two structurally related kinases, designated IKK $\alpha$  and IKK $\beta$ , and one adapter molecule, designated IKK $\gamma$  or NEMO (9). Gene-targeting studies showed essential roles of IKK $\beta$  and IKK $\gamma$ /NEMO in cytokine-induced NF- $\kappa$ B activation (10–15). However, it remains to be determined how receptor-mediated signals finally activate IKK. These studies also demonstrated a critical role of NF- $\kappa$ B in the protection of cells from TNF-induced cell death, although the molecular mechanism is not completely understood.

The TRAFs were identified originally as signal-transducing molecules for the TNF-R and the IL-1 receptor superfamilies (16, 17). *Ex vivo* data demonstrated that TRAF2, TRAF5, and TRAF6 activate NF- $\kappa$ B and are involved in NF- $\kappa$ B activation through these receptors (16, 17). Previous studies suggested that TRAFs interact with NF- $\kappa$ B-inducing kinase (NIK), MAP kinase/ERK kinase 1 (MEKK1), transforming growth factor  $\beta$ -activated kinase, or atypical protein kinase C, and these kinases phosphorylate IKKs resulting in NF- $\kappa$ B activation (18–21). However, the exact contribution of these kinases to cytokine-induced NF- $\kappa$ B activation is not yet clear. So far, RIP and TRAF6 have been shown to play essential roles in TNF- and IL-1/lipopolysaccharide-induced NF- $\kappa$ B activation, respectively (22–24). In contrast, TRAF2- or TRAF5-deficient mice did not show substantial defects in TNF-induced NF- $\kappa$ B activation (25, 26), suggesting that TRAF2 and TRAF5 are not essential or play a redundant role in TNF-induced NF- $\kappa$ B ac-

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<sup>1</sup> The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; TRAF, TNF receptor-associated factor; TRADD, TNFR1-associated death domain protein; RIP, receptor-interacting protein; MEF, murine embryonic fibroblast; JNK, c-Jun N-terminal kinase; IKK, I $\kappa$ B kinase; IAP, inhibitor of apoptosis; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NIK, NF- $\kappa$ B-inducing kinase; MEKK1, mitogen-activated protein (MAP) kinase/extracellular signal-related kinase (ERK) kinase 1; IL, interleukin; DKO, double knockout; mAb, monoclonal antibody; GST, glutathione S-transferase; HA, hemagglutinin; CHX, cycloheximide.

tivation. To further examine the contribution of TRAF2 and TRAF5 to TNF-induced NF- $\kappa$ B activation, we generated TRAF2 and TRAF5 double knockout (DKO) mice and characterized the response to TNF. We found redundant or nonredundant roles for TRAF2 and TRAF5 in TNF-induced NF- $\kappa$ B activation, JNK activation, and protection from cell death.

#### EXPERIMENTAL PROCEDURES

**Reagents and Cell Culture**—Recombinant human TNF, murine TNF, and murine IL-1 $\beta$  were purchased from BD Pharmingen. Anti-HA (12CA5) and anti-Flag (M2) monoclonal antibodies (mAbs) were from Roche Molecular Biochemicals and Sigma, respectively. Anti-Myc mAb (9E10) was obtained from ATCC. Anti-RIP mAb was purchased from Transduction Laboratories. Anti-IKK $\alpha$ , anti-JNK1, and anti-I $\kappa$ B $\alpha$  antibodies were purchased from Santa Cruz Biotechnology. HEK293 cells and murine embryonic fibroblasts (MEFs) were cultured in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

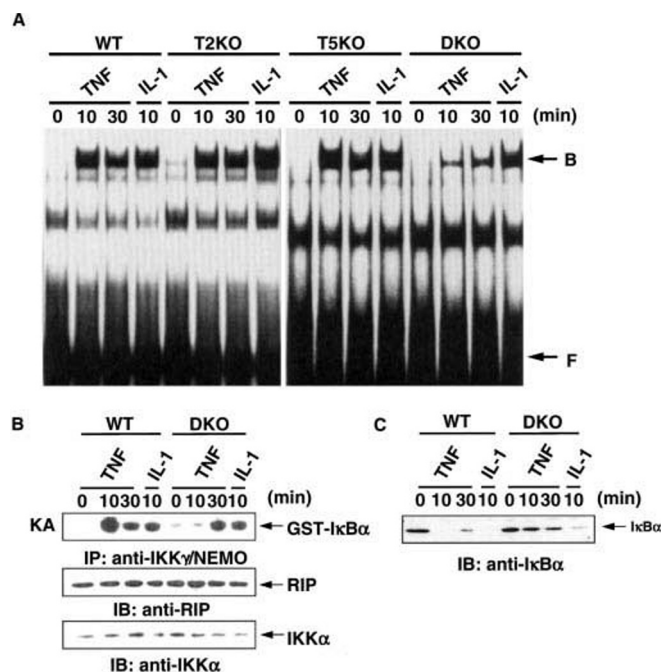
**Plasmids**—pcDNA3-HA-TRAF2, pRK5-Flag-RIP, and pRK5-Myc-TRADD were described previously (5, 27). Human TRAF5 was subcloned into pCR-HA vector, designated pCR-HA-TRAF5.

**Generation of *traf2*<sup>-/-</sup>*traf5*<sup>-/-</sup> (DKO) Mice**—To maximize the yield of double mutant mice, we first generated *traf2*<sup>+/-</sup>*traf5*<sup>-/-</sup> and *traf2*<sup>+/-</sup>*traf5*<sup>+/-</sup> mice by crossing *traf2*<sup>+/-</sup> and *traf5*<sup>-/-</sup> mice. We then crossed *traf2*<sup>+/-</sup>*traf5*<sup>+/-</sup> and *traf2*<sup>+/-</sup>*traf5*<sup>-/-</sup> mice to generate *traf2*<sup>-/-</sup>*traf5*<sup>-/-</sup> mice. Genotypic analysis of these mice was performed as described previously (25, 26).

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay was performed essentially as described previously (28). Briefly, MEFs ( $1 \times 10^6$ ) were stimulated with murine TNF (10 ng/ml) or IL-1 $\beta$  (10 ng/ml) for the indicated time periods. Then, the cells were washed with ice-cold phosphate-buffered saline and harvested. The cells were treated with 0.5% Nonidet P-40, and the nuclear extracts were prepared. The nuclear extracts (5  $\mu$ g) were incubated with a <sup>32</sup>P-labeled NF- $\kappa$ B-specific oligonucleotide probe containing two tandemly positioned NF- $\kappa$ B-binding sites from the HIV-1 enhancer. Reactions were subjected to 6% polyacrylamide gel electrophoresis and analyzed on a Fuji BAS2500 image analyzer.

**In Vitro Kinase Assay**—*In vitro* kinase assay was performed essentially as described previously (29). Briefly, MEFs ( $4 \times 10^6$  for IKK assay;  $1 \times 10^6$  for JNK assay) were plated in 150- or 100-mm dishes. Then the cells were stimulated with murine TNF (10 ng/ml) or IL-1 $\beta$  (10 ng/ml) for the various times, and the reaction was stopped with ice-cold phosphate-buffered saline and lysed in 1 ml of a lysis buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.3), 250 mM NaCl, 1 mM EDTA, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM NaF. Nuclei were removed by centrifugation, and the supernatants were immunoprecipitated with anti-IKK $\gamma$ /NEMO antibody (30) or anti-JNK1 antibody and protein G-Sepharose. The immunoprecipitates were washed three times with the lysis buffer and twice with the kinase buffer containing 20 mM HEPES (pH 7.3), 150 mM NaCl, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM dithiothreitol. The immunoprecipitates were then incubated with 1  $\mu$ g of GST-I $\kappa$ B $\alpha$  (1–100) for IKK assay or GST-c-Jun(1–79) for JNK assay and [ $\gamma$ -<sup>32</sup>P]ATP (10  $\mu$ Ci) in the kinase buffer for 20 min at 30 °C. The reaction was stopped by the addition of Laemmli's sample buffer. The eluted proteins were subjected to 12% SDS-polyacrylamide gel electrophoresis, and the autoradiograms were visualized on an image analyzer (Fujix, BAS2500). In all cases expression of IKK $\alpha$ , RIP, or JNK1 was verified by immunoblotting of aliquots of the cell lysates as described below.

**Co-immunoprecipitation and Western Blotting**—Co-immunoprecipitation and Western blotting were performed as described previously (31). Briefly, 293 cells ( $4 \times 10^6$ ) were transiently transfected with the indicated expression vectors. Twenty-four hours after transfection, the cells were harvested and lysed in a lysis buffer containing 0.5% Nonidet P-40, 50 mM Tris (pH 7.4), 250 mM NaCl, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Then, one-half of the lysates was immunoprecipitated with control IgG, anti-Flag mAb, or anti-Myc mAb followed by the addition of 30  $\mu$ l of protein G-Sepharose. The precipitates were washed with lysis buffer, and the eluates were subjected to 10% SDS-polyacrylamide gel electrophoresis followed by transfer onto polyvinylidene difluoride membrane (Millipore). The membrane was incubated with biotin-conjugated anti-HA mAb followed by avidin-biotinylated peroxidase complex (Vectastain). The signal was detected using an enhanced chemilumines-



**FIG. 1. Impaired NF- $\kappa$ B and JNK activation by TNF in DKO MEFs.** A, wild-type (WT), T2KO, T5KO, and DKO MEFs ( $1 \times 10^6$ ) were stimulated with TNF (10 ng/ml) or IL-1 $\beta$  (10 ng/ml) for the indicated time periods. Nuclear extracts (5  $\mu$ g) were prepared and incubated with <sup>32</sup>P-labeled double-stranded  $\kappa$ B oligonucleotide. B and F indicate the positions of the bound and free fractions, respectively. B, *in vitro* kinase assay of the IKK complex. Wild-type and DKO MEFs ( $4 \times 10^6$ ) were stimulated with TNF (10 ng/ml) or IL-1 $\beta$  (10 ng/ml) for the indicated time periods. The IKK complex was immunoprecipitated from the lysates with anti-IKK $\gamma$ /NEMO antibody, and the kinase activity (KA) was assayed using GST-I $\kappa$ B $\alpha$ (1–100) as a substrate. The levels of RIP and IKK $\alpha$  were verified by Western blotting with anti-RIP and anti-IKK $\alpha$  antibodies. IP, immunoprecipitate; IB, immunoblot. C, degradation of I $\kappa$ B $\alpha$ . After the stimulation as described in B, the levels of I $\kappa$ B $\alpha$  were examined by Western blotting with anti-I $\kappa$ B $\alpha$  antibody.

cence (ECL) Western blotting Detection System Plus (Amersham Pharmacia Biotech) according to the manufacturer's instruction. Expression of the transfected proteins was verified by subjecting aliquots of total lysates to immunoblotting with the indicated anti-tag mAbs.

**Cell Death Assay**—MEFs ( $5 \times 10^3$ /well) were plated onto 96-well plates and cultured for 12 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Then the cells were incubated with various amounts of murine TNF in the presence or absence of 0.25  $\mu$ g/ml cycloheximide (CHX, Sigma) for 24 h. Cell viability was determined by WST assay using a cell counting kit (Dojindo).

#### RESULTS

**TNF-induced NF- $\kappa$ B Activation Is Severely Impaired in *traf2*<sup>-/-</sup>*traf5*<sup>-/-</sup> (DKO) MEFs**—Taken that the cells from *traf2*<sup>-/-</sup> (T2KO) or *traf5*<sup>-/-</sup> (T5KO) mice did not show a substantial defect in TNF-induced NF- $\kappa$ B activation (25, 26), TRAF2 and TRAF5 might play a redundant role in the NF- $\kappa$ B activation by TNF. To explore this possibility, we generated TRAF2 and TRAF5 DKO mice by crossing T2KO and T5KO mice. The percentage of DKO pups at birth was lower than the expected Mendelian ratio, and these pups became runted and died within 2 to 3 weeks. A detailed phenotype of DKO mice will be published elsewhere. To investigate whether NF- $\kappa$ B activation was impaired in DKO mice, we prepared MEFs from E13.5–E14.5 embryos. We first examined nuclear translocation of NF- $\kappa$ B by electrophoretic mobility shift assay. As reported previously (25, 26), TNF-induced NF- $\kappa$ B translocation was not significantly impaired in either T2KO or T5KO MEFs (Fig. 1A). In contrast, DKO MEFs showed severe impairment of nuclear translocation of NF- $\kappa$ B upon TNF stimulation, whereas upon IL-1 stimulation this was intact (Fig. 1A). These results sug-



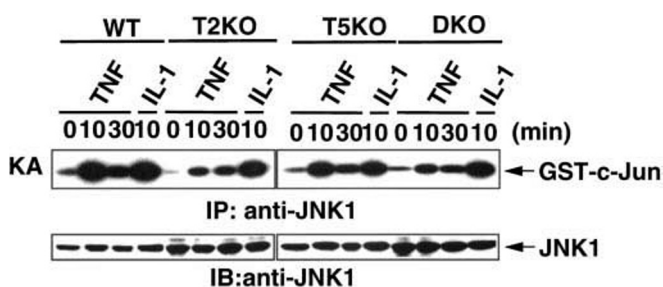


FIG. 2. **TNF- and IL-1-induced JNK activation.** MEFs ( $1 \times 10^6$ ) were stimulated with TNF (10 ng/ml) or IL-1 $\beta$  (10 ng/ml) for the indicated time periods. The lysates were immunoprecipitated with anti-JNK1 antibody, and kinase activity (KA) was assayed using GST-c-Jun(1–79) as a substrate. The levels of JNK1 in total cell lysates were verified by Western blotting with anti-JNK1 antibody. IP, immunoprecipitate; IB, immunoblot.

gested a redundant role of TRAF2 and TRAF5 in TNF-induced NF- $\kappa$ B activation.

Nuclear translocation of NF- $\kappa$ B requires phosphorylation and subsequent degradation of I $\kappa$ Bs. We next assessed the kinase activity of IKK to phosphorylate I $\kappa$ B $\alpha$ . Wild-type and DKO MEFs were stimulated with TNF or IL-1 for 10 or 30 min, and the lysates were immunoprecipitated with anti-IKK $\gamma$ /NEMO antibody. The precipitates were subjected to *in vitro* kinase assay using GST-I $\kappa$ B $\alpha$  as a substrate. As shown in Fig. 1B, phosphorylation of GST-I $\kappa$ B $\alpha$  induced by TNF, but not IL-1, at 10 min was dramatically reduced in DKO MEFs as compared with wild type. The late appearance of the kinase activity at 30 min might explain the residual NF- $\kappa$ B binding activity observed in DKO MEFs. We also examined the degradation of I $\kappa$ B $\alpha$  by Western blotting. In wild-type MEFs, I $\kappa$ B $\alpha$  completely disappeared at 10 min and reappeared at 30 min after TNF stimulation (Fig. 1C). In contrast, I $\kappa$ B $\alpha$  levels were only partially reduced at 10 and 30 min after TNF stimulation in DKO MEFs (Fig. 1C). On the other hand, IL-1-induced degradation of I $\kappa$ B $\alpha$  was comparable between wild-type and DKO MEFs. These results suggested that TRAF2 and TRAF5 play a critical role in TNF-induced NF- $\kappa$ B activation by mediating rapid activation of IKK and degradation of I $\kappa$ B $\alpha$ .

**TNF-induced JNK Activation in DKO MEFs**—Previous studies showed a severe impairment of JNK activation by TNF in T2KO MEFs but not T5KO MEFs (25, 26). We next examined TNF-induced JNK activation in DKO MEFs. MEFs were stimulated with TNF or IL-1 for 10 or 30 min, and the lysates were immunoprecipitated with anti-JNK antibody. The precipitates were subject to *in vitro* kinase assay using GST-c-Jun as a substrate. JNK activation by TNF, but not IL-1, was significantly reduced in T2KO and DKO MEFs as compared with wild-type and T5KO MEFs (Fig. 2). The reduction of JNK activity in T2KO MEFs was almost comparable with that in DKO MEFs, suggesting that TRAF5 does not substantially contribute to TNF-induced JNK activation.

**TRAF5 Interacts Physically with RIP**—To further investigate the molecular mechanism by which TRAF5 is involved in TNF-induced NF- $\kappa$ B activation, we examined whether TRAF5 interacts physically with TNF receptor-associated signaling molecules such as TRADD and RIP (4, 5, 22). To address this possibility, 293 cells were transiently transfected with Flag-tagged RIP or Myc-tagged TRADD along with HA-tagged TRAF2 or TRAF5. Cell lysates were immunoprecipitated with anti-Flag or anti-Myc antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-HA antibody. As reported previously (4, 5), TRAF2 was efficiently co-immunoprecipitated with RIP and TRADD (Fig. 3). TRAF5 was also co-immunoprecipitated with RIP but not TRADD (Fig. 3).

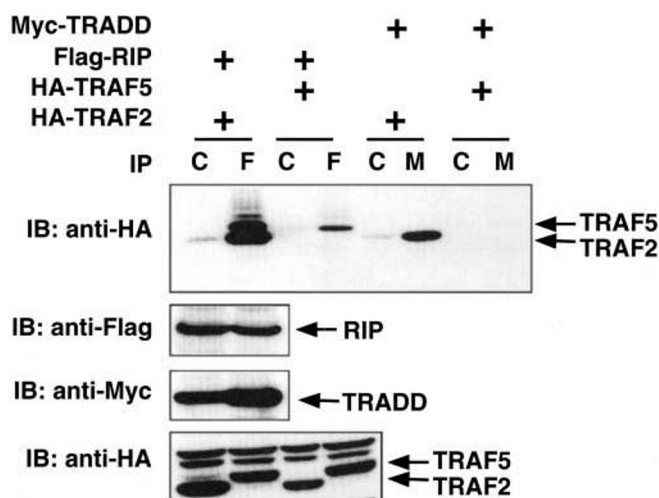


FIG. 3. **Interaction of TRAF5 with RIP but not with TRADD.** HEK293 cells ( $4 \times 10^6$ ) were transiently transfected with 2.5  $\mu$ g each of the indicated expression vectors. After 24 h, the cell extracts were prepared and immunoprecipitated with control (C), anti-Flag (F), or anti-Myc (M) mAb. Then the immunoprecipitates were analyzed by immunoblotting with biotin-conjugated anti-HA antibody (top panel). Expression of each construct was verified by Western blotting using total cell lysates with the corresponding anti-tag antibodies (lower three panels). The positions of RIP, TRADD, TRAF5, and TRAF2 are indicated by the arrows. IP, immunoprecipitate; IB, immunoblot.

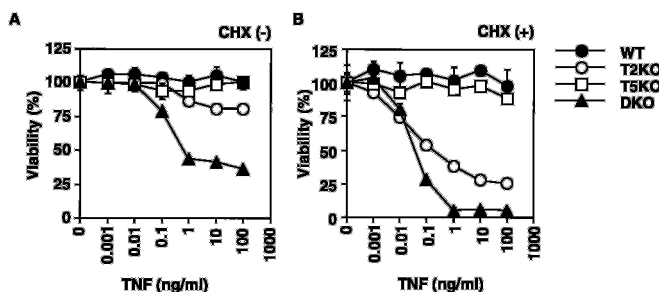


FIG. 4. **High sensitivity of DKO MEFs to TNF-induced cytotoxicity.** MEFs ( $5 \times 10^3$ ) were incubated with various amounts of TNF in the absence (A) or presence of CHX (0.25  $\mu$ g/ml) (B) for 24 h. Cell viability was determined by WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). Data are shown as the mean  $\pm$  S.D. of triplicate samples. Similar results were obtained with three independently established MEFs from DKO mice. WT, wild type.

These results suggested that TRAF5 participates in the TNF-induced NF- $\kappa$ B activation via interaction with RIP.

**Increased Susceptibility of DKO MEFs to TNF-induced Cell Death**—We next examined the susceptibility of wild-type, T2KO, T5KO, and DKO MEFs to TNF-induced cell death in the presence or absence of a protein synthesis inhibitor, CHX. As reported previously (25), T2KO MEFs were only slightly susceptible to TNF-induced cell death in the absence of CHX (Fig. 4A) but was highly susceptible in the presence of CHX (Fig. 4B). In contrast, the susceptibility of T5KO MEFs was almost comparable with that of wild-type MEFs in the absence or presence of CHX. Notably, DKO MEFs were highly susceptible to TNF-induced cell death even in the absence of CHX (Fig. 4A) and were more susceptible than T2KO MEFs in the presence of CHX (Fig. 4B). These results suggested a redundant but critical role of TRAF2 and TRAF5 in protecting MEFs from TNF-induced cell death, which is dependent on protein synthesis, and a predominant role of TRAF2 in the absence of protein synthesis.

## DISCUSSION

In the present study, we investigated the relative contributions of TRAF2 and TRAF5 to TNF-induced NF- $\kappa$ B activation, JNK activation, and protection from TNF-induced cell death by utilizing MEFs from T2KO, T5KO, and DKO mice. We found redundant or nonredundant roles for TRAF2 and TRAF5 in these TNF responses.

We and others previously showed that TRAF5, as well as TRAF2, is used by multiple members of the TNF receptor superfamily (16). Thus, we first speculated that TRAF5 is also involved in the TNF-R-mediated signaling pathway and can substitute for TRAF2 in NF- $\kappa$ B activation by TNF. We demonstrated that NF- $\kappa$ B activation by TNF, but not IL-1, was substantially reduced in DKO MEFs (Fig. 1). Moreover, we also showed that TRAF5 physically interacts with RIP (Fig. 3), an essential component of TNF-induced NF- $\kappa$ B activation (22, 32). In contrast to RIP-deficient cells (22, 32), significant levels of TNF-induced NF- $\kappa$ B binding activity (Fig. 1A) and IKK activity (Fig. 1B) were observed in DKO MEFs, especially at a later time point. This suggests that some molecule other than TRAF2 and TRAF5 may interact with RIP and mediate NF- $\kappa$ B activation. One possible candidate is p62, which interacts with RIP and activates atypical protein kinase C, resulting in IKK activation (33).

Recent studies have demonstrated that RIP interacts directly with IKK $\gamma$ /NEMO and plays a critical role in the recruitment of the IKK complex to TNF-R1 (34, 35). However, the exact mechanism by which TRAFs activate NF- $\kappa$ B remains obscure. Initial studies have suggested that NIK or MEKK1 is involved in TNF-induced NF- $\kappa$ B activation (18, 36). However, neither NIK- or MEKK1-deficient mice showed a substantial defect in TNF-induced NF- $\kappa$ B activation (37–39), suggesting that these kinases are not essential or play redundant roles in TNF-induced NF- $\kappa$ B activation. Generation of NIK and MEKK1 double deficient mice will be required to address this issue. Alternatively, TRAF2 and TRAF5 may activate other signaling pathways independent of these kinases. A recent paper demonstrated that TRAF6 has ubiquitin ligase activity, which is required for TRAF6-mediated activation of IKK (40).

We have previously demonstrated a severe defect in TNF-induced JNK activation in T2KO MEFs (25). In contrast to TNF-induced NF- $\kappa$ B activation in DKO MEFs, there was no additional defect in TNF-induced JNK activation in DKO MEFs as compared with T2KO MEFs (Fig. 2). Although overexpression of TRAF5 could activate JNK, the ability of TRAF5 to activate JNK and the dominant negative effect of truncated TRAF5 on JNK activation were relatively weaker than those of TRAF2 (31). Altogether, TRAF5 seems to play a greater role in TNF-induced NF- $\kappa$ B activation than in JNK activation under physiological conditions.

Our present examination of the susceptibility of T2KO, T5KO, and DKO MEFs to TNF-induced cell death has revealed both redundant and nonredundant roles of TRAF2 and TRAF5 in protection from TNF-induced cell death. In the absence of CHX, DKO but not T2KO or T5KO MEFs were highly susceptible to TNF-induced cell death (Fig. 4A). In the presence of CHX, both T2KO and DKO but not T5KO MEFs were highly susceptible to TNF-induced cell death (Fig. 4B). Collectively, these data suggested that TRAF2 and TRAF5 may redundantly mediate NF- $\kappa$ B-dependent anti-apoptotic pathway, which is dependent on protein synthesis. TRAF2 may also mediate an NF- $\kappa$ B-independent anti-apoptotic pathway, which is not dependent on protein synthesis.

Previous studies reported that expression of XIAP, c-IAP1, c-IAP2, and A1/Bfl-1 was induced by TNF in an NF- $\kappa$ B-depend-

ent manner in various types of cells (41–44). Although our RNase protection assay and Northern blot analysis showed that induction of A1/Bfl-1 was severely impaired in DKO MEFs, stable transfection of A1/Bfl-1 did not fully protect DKO MEFs from TNF-induced cell death (data not shown). These results suggest that some molecule(s) other than A1/Bfl-1 induced by NF- $\kappa$ B may be primary responsible for TRAF-mediated NF- $\kappa$ B-dependent protection from TNF-induced cell death. Further studies are now under way to identify such molecules.

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